Title: High Infection Rates in Adult Macaques Following Intravaginal or Intrarectal Zika Virus Inoculation

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ABSTRACT Unprotected sexual intercourse between persons residing or traveling from regions with Zika virus (ZIKV) transmission is a risk factor for infection. To model Zika virus infection risk following sexual intercourse, we experimentally inoculated groups of rhesus and cynomolgus macaques with ZIKV either by the intravaginal or intrarectal route. In those macaques inoculated intravaginally, we detected viremia in 50% of both species followed by seroconversion. Viremia was detected in 75% of rhesus and 100% cynomolgus macaques inoculated intrarectally, followed by seroconversion in all exposed macaques. Our results suggest that transmission of ZIKV via sexual intercourse may serve as a virus maintenance mechanism in the absence of mosquito-to-human transmission and could increase the probability of ZIKV establishment and spread in naïve regions.

Zika virus (ZIKV) is a member of the Spondweni serogroup, genus *Flavivirus*, family *Flaviviridae* (1, 2). Since its isolation in 1947 (3), intermittent reports of ZIKV infection have been described throughout sub-Saharan Africa and Southeast Asia (4). Recently ZIKV extended its geographic distribution into naïve regions, resulting in large outbreaks in the tropics (5-7). The majority of ZIKV infections are asymptomatic and those that are symptomatic typically present with a mild febrile illness (2, 5-7). However, severe clinical outcomes including congenital birth defects and Guillian-Barré syndrome have been reported in a subset of infections (2, 5-8). While the primary mechanism of ZIKV transmission is through the bite of an infective mosquito (3, 9, 10), sexual transmission involving virus strains originating from both the African and Asian ZIKV lineages have been reported (11-18). This route of transmission has been identified in non-traveling female and male sexual partners of men who were infected with ZIKV during travel to ZIKV-endemic regions (11-15, 17-19).

Recent evidence suggests that the sexual transmission of ZIKV is responsible for a substantial number of infections (17-19) and could be a maintenance mechanism in the absence of mosquito-to-human transmission, as well as a mechanism by which ZIKV is introduced to naïve regions. Viral persistence studies have demonstrated the isolation of infectious ZIKV from the ejaculate of a vasectomized patient 69 days post-illness (20), detected ZIKV RNA in the spermatozoa of another patient 56 days post-illness (21), and detected ZIKV RNA in semen specimens for at least six months following illness (22, 23). While the titer of infectious ZIKV in the semen remains unknown, RNA levels up to 7.5-8.6 $\log_{10}$ copies/mL have been reported (13, 21, 24, 25). These data suggest that male-to-female vaginal, male-to-female rectal and male-to-male rectal transmission may occur more often than previously recognized and that persons may

be exposed to a higher ZIKV dose from sexual intercourse with an infectious male than through the bite of an infective mosquito (26, 27).

To model ZIKV infection risk following sexual intercourse, we non-traumatically administered 7.0 $\log_{10}$ PFU/mL of the ArD 41525 ZIKV isolate to the vaginal canal or rectum of 16 adult rhesus or cynomolgus macaques and monitored for evidence of infection through 28 days post-inoculation (DPI). This dose was selected to correspond to high ZIKV RNA load(s) reported in human semen (13, 21, 24, 25).

MATERIALS AND METHODS

Study design and data analyses. This was a pilot study designed to determine if non-human primates (NHPs) are susceptible to ZIKV infection via the intravaginal and intrarectal routes. Sample size estimates for the two study groups (rhesus and cynomolgus macaques) were based on historic reports of ZIKV experimental infections involving NHPs (3, 10, 28, 29). Power analysis with the type I error rate set to 0.05 indicated that a group size of four individuals had an 80% probability to detect ZIKV infection following intravaginal or intrarectal inoculation with the virus. This study was not designed to have the statistical power to perform analyses of chemistry, hematology and/or temperature data. Investigators were not blinded during the course of the study.

Non-human primates. Research was conducted under an Institutional Animal Care and Use Committee (IACUC)-approved protocol at United States Army Medical Research Institute for Infectious Diseases (USAMRIID). This protocol complied with the Animal Welfare Act, Public

Health Service Policy, and other Federal statutes and regulations relating to animals and experiments involving animals. USAMRIID is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC) and adheres to principles stated in the Guide for the Care and Use of Laboratory Animals, National Research Council (2011).

Four female rhesus macaques of Chinese origin (R1, R2, R3 and R4) and four female cynomolgus macaques of Cambodian origin (C1, C2, C3 and C4) were individually housed (age range: 8.5 to 9.3 years) during the intravaginal inoculation experiment. For the intrarectal inoculation experiment an additional four rhesus macaques (two male and two female) of Chinese origin R5 (male), R6 (female), R7 (male) and R8 (female); and four cynomolgus macaques (two male and two female) of Cambodian origin C5 (female), C6 (female), C7 (male) and C8 (male) were individually housed (age range: 8.2 to 11.4 years). All macaques were prescreened and determined to be negative for ZIKV, Herpes B virus, Simian-T-lymphotropic virus-1, Simian immunodeficiency virus, Simian retrovirus 1/2/3 antibodies, and tuberculosis, Salmonella, Campylobacter, Hypermucoid HVM Klebsiella, and Shigella infections.

Virus isolate. The ArD 41525 ZIKV isolate was used in this study was kindly provided by Drs. Robert Tesh and Scott Weaver at the University of Texas Medical Branch. This isolate was made from a pool of *Aedes africanus* mosquitoes collected in Eastern Senegal in 1984 (passage history: AP61#1, C6/36#1, Vero #3) and was previously sequenced (Genbank Accession: KU955591) (30). We selected the ArD 41525 isolate due to its low passage history and the ancestral nature of the African lineage (4, 31). Although ZIKV sequences comprise at least two

phylogenetic lineages, African and Asian, these lineages constitute a single virus serotype (1, 4, 32-34). Furthermore, male-to-female sexual transmission of ZIKV has involved virus strains originating from both ZIKV lineages (11-18). Prior to the initiation of this study, virus challenge stocks were confirmed to be free of mycoplasma and passage associated mutations (4).

Intravaginal virus inoculation. Anesthetized macaques were placed in dorsal recumbency with their hips elevated above their torso at a 30˚ angle and 3 to 5 cm of a lubricated size 7FR infant feeding tube (Mallinckrodt Pharmaceuticals, St. Louis, MO, USA) was inserted into the vaginal opening. A 3-mL syringe containing 7.0 $\log_{10}$ PFU/mL of cell-free ZIKV suspended in 2 mL of PBS was connected to the end of the infant feeding tube and slowly administered. A 500 µL flush of 0.9% sodium chloride (BD, Franklin Lakes, NJ, USA) was used to insure that all ZIKV inoculum was administered to the vagina. Macaques stayed in dorsal recumbency with hip elevation for at least 20 minutes: R1 (26 min); R2 (23 min); R3 (21 min); R4 (20 min); C1 (21 min); C2 (30 min); C3 (28 min); and C4 (24 min).

Intrarectal virus inoculation. Anesthetized macaques were placed in an inverted Trendelenburg position (25˚ to 30˚ down angle) and 3 to 5 cm of a lubricated size 7FR infant feeding tube (Mallinckrodt Pharmaceuticals, St. Louis, MO, USA) was inserted into the rectum. A 10 mL 0.9% sodium chloride flush was slowly administered to soften impacted fecal material lining the rectum. Following the 0.9% sodium chloride flush, 7.0 $\log_{10}$ PFU/mL of cell-free ZIKV suspended in 2 mL of PBS was slowly administered, followed by a 500 µL flush of 0.9% sodium chloride to insure that all ZIKV inoculum was administered to the rectum. Macaques stayed in an

inverted Trendelenburg position for at least 15 minutes: R5 (15 min); R6 (15 min); R7 (23 min); R8 (17 min); C5 (20 min); C6 (21 min); C7 (20 min); and C8 (20 min).

Observations and blood collections. Following virus exposure, macaques were evaluated daily for signs of illness; vital signs and input/output were noted. Blood collections and physical examinations including weight and rectal temperature were conducted under anesthesia on days: -7, 1-7, 9, 12, 15, 21 and 28. Menstruation patterns were not recorded prior to day 0. Menstruation was noted during daily observations (days 0-28), but may have occurred on additional days (e.g. light or transient events).

Serum chemistry and hematology. Comprehensive metabolic panels were performed on serum collected in 2.5 ml Z Serum Separator Clot Activator VACUETTE® tubes (Greiner Bio-One, Monroe, NC, USA) using a Piccolo Xpress Chemistry Analyzer and Piccolo General Chemistry 13 Panel (Abbott Point of Care, Princeton, NJ, USA). Complete blood counts were performed on whole blood collected in S-Monovette® 1.2 ml, K3 EDTA tubes (Sarstedt, Nümbrecht, Germany) on a CELL-DYN 3700 system (Abbott Point of Care).

Telemetry devices and monitoring. Prior to the study, macaques were surgically implanted with T27F-1B radio telemetry devices (Konigsberg Instruments, Pasadena, CA, USA; telemetry instrument in macaque C4 failed). Notocord-hem Evolution software platform (Version 4.3.0.47, Notocord Inc., Newark, NJ, USA) was used to capture and analyze data. Temperature data points were averaged and statistically filtered to remove noise and signal artifacts to generate a single data point every 30 seconds.

Infectious virus quantification. Virus titration was performed on confluent Vero cell (CCL-81, ATCC, Manassas, VA, USA) monolayers in six-well plates by plaque assay. Duplicate wells were infected with 0.1 mL aliquots of serial 10-fold diluted virus in growth medium comprised of Dulbecco's Modified Eagle Medium (Corning Life Sciences, Tewksbury, MA, USA), supplemented with 50 µg/mL gentamicin (Gibco, Carlsbad, CA, USA), 1.0 mM sodium pyruvate, 1% v/v non-essential amino acids (Sigma Aldrich, St. Louis, MO, USA) and 0.4 mL of growth medium. Virus was allowed to absorb for 1 hr at 37°C and was then removed prior to overlaying the cell monolayers with 3 mL of 1% w/v Sea-Plaque agarose (Cambrex Bio Science, East Rutherford, NJ, USA) in growth medium. Cells were incubated at 37°C (5% CO_2) for 4-5 days and were then fixed with 4% formaldehyde (Fisher Scientific, Waltham, MA, USA) in phosphate buffered saline (PBS; Corning Life Sciences) for 24 hrs. Following removal of the overlay, the cell monolayers were stained with 2% crystal violet (Sigma Aldrich) in 70% methanol (Sigma Aldrich) for 5-10 min at ambient temperature and excess stain was removed under running water. Plaques were counted and the results were reported as the number of plaque forming units (PFU)/mL, with a lower limit of detection of 1.0 $\log_{10}$ PFU/mL.

Serology. Plaque reduction neutralization tests (PRNTs), considered the gold standard for clinical diagnosis of past infection, were performed to determine pre-and post-exposure immune responses (35, 36). Serum samples were heat-inactivated at 56°C (30 min). Samples were serially diluted 2-fold in PBS, mixed with an equal volume of 2000 PFU/mL of ZIKV and incubated for 1h at 37°C (5% CO_2). Confluent Vero cell monolayers in 6-well plates were inoculated with 100µL of serum/virus mixture in triplicate. Plates were incubated at 37°C (5% CO_2) for 5 days

and fixed and then stained with crystal violet as described above. $PRNT_{80}$ titers were calculated and expressed as the reciprocal of serum dilution yielding a >80% reduction in the number of plaques. Pre-exposure sera collected from the rhesus macaques at -28 DPI and from the cynomolgus macaques at -20 DPI showed no ZIKV neutralization activity, indicating that these animals were not previously exposed to the virus. Post-exposure sera were screened on days 7, 15, 21 and 28.

RESULTS

Intravaginally inoculated macaque viremias and antibody responses. Following intravaginal ZIKV inoculation, 50% of both rhesus (2/4) and cynomolgus (2/4) macaques had detectable viremias, with mean peak titers of 3.8 $\log_{10}$ PFU/mL and 3.5 $\log_{10}$ PFU/mL, respectively (Table 1). Viremia was detected between 4 and 6 DPI in rhesus macaques (mean duration: 3.0 days) and between 3 and 7 DPI in cynomolgus macaques (mean duration: 4.0 days). By 15 DPI, only those rhesus and cynomolgus macaques that exhibited viremia seroconverted (R1, R4, C3 and C4), as evidenced by $PRNT_{80}$ titers greater than 1:640 (Table 1). No neutralization was observed in macaques R2, R3, C1 and C2 (Table 1). All macaques were observed to menstruate during the course of the study, but none were observed to be menstruating at the time of virus inoculation.

Intrarectally inoculated macaque viremias and antibody responses. Following intrarectal ZIKV inoculation, 75% of rhesus (3/4) and 100% of cynomolgus (4/4) macaques had detectable viremias (R5, R7, R8, C5, C6, C7, C8), with mean peak titers of 4.8 $\log_{10}$ PFU/mL for both species (Table 2). Two cynomolgus macaques (C5, C8) had viremias $\geq$ 5.0 $\log_{10}$ PFU/mL for two days. Viremia was detected between 3 and 7 DPI in rhesus macaques (mean duration: 3.0

days) and between 2 and 6 DPI in cynomolgus macaques (mean duration: 2.8 days). However, by 15 DPI all rhesus and cynomolgus macaques seroconverted (R5, R6, R7, R8, C5, C6, C7, C8), as evidenced by $PRNT_{80}$ titers greater than 1:640 (Table 2).

Clinical signs and laboratory results. No overt clinical signs including pyrexia, joint swelling, and weight loss or decreased appetite were observed in any of the infected macaques. Potentially clinically significant marked increases or decreases in laboratory values lasting more than one day in infected macaques were: GLU (R1, R4, R6), BUN (R4, R8), TP (R7), ALT (R6, C3, C4, C5, C6, C7, C8), AST (R4, R6, C3, C4, C5, C7, C8), ALP (C6), TBIL (R8), GGT (C8), AMY (R1, R4, C3), WBC (R5), RBC (C3, C4, C6), PLT (R1, R6), %NEUT (R1, R4, R6), %LYMPH (R1, R4, R6), %MONO (R1, R4, R5, R8, C3, C4, C5, C6), %BASO (R1, R4, R6), %EOS (R1, R5, R8, C3, C4) (Supplementary Figures1 and 2). Macaques observed to menstruate during the study were: R1 (days 4, 5, 6); R2 (days 1, 2); R3 (day 2); R4 (days 9); C1 (8, 9, 10); C2 (day 15); C3 (days 14, 15); and C4 (day 7).

DISCUSSION

Sexual transmission of ZIKV is underestimated and its detection is confounded in those regions with active mosquito-to-human virus transmission (17-19). In an effort to gauge the likelihood of infection following exposure via vaginal or anal intercourse, we inoculated the vaginal canal or rectum of rhesus and cynomolgus macaques with ZIKV. Intravaginal and intrarectal exposure resulted in infection in absence of clinical disease, followed by seroconversion in both species. The magnitude and duration of detectable viremia following intravaginal and intrarectal inoculation indicates that NHPs, as well as humans, could infect primary mosquito vector

species. Although the infectious dose 50 required for primary urban and sylvatic mosquito ZIKV vectors to become infected and transmit infectious virus remains unknown, other flavivirus-vector host systems have demonstrated mosquito transmission following low-dose experimental exposure (undetectable to < 3.0 $\log_{10}$ PFU/mL) (26, 37-39). Moreover, our results suggest that sexual transmission may extend the duration of the current ZIKV epidemics and increase the probability of ZIKV introduction and establishment in naïve regions. Likewise, sexual transmission among NHPs may be a secondary mechanism by which ZIKV is maintained in an enzootic cycle.

Despite the presence of viremia in intravaginally and intrarectally exposed macaques, overt clinical signs including pyrexia, joint swelling, and weight loss or decreased appetite were not observed in our study. The duration of viremia and clinical signs in NHPs following ZIKV infection resulting from a mosquito bite(s), or by intracranial or subcutaneous inoculation of either African or Asian ZIKV isolates vary in the reported literature (3, 10, 28, 29, 40-46). Although sentinel or experimentally ZIKV-infected NHPs have exhibited fever or an elevated temperature (3, 28, 42, 43), or decreased appetite and weight loss (40); other experimental NHP ZIKV infections report a lack of overt clinical illness (3, 10, 28, 29, 41), which are consistent with our study results and the majority of human ZIKV infections (2, 5-7). In our study, transient increases or decreases in WBC, %NEUT, %LYMPH, %MONO and GGT were the only parameters that were observed solely in infected macaques. Analogous to our observations, recent studies have also reported a rise in AST (40, 42, 43), ALT (40, 42, 43) and %MONO (43) in ZIKV-infected macaques. While elevations in some laboratory values may be a result of repeated daily anesthesia (47), studies with increased numbers are needed to resolve which

clinical laboratory parameters are associated with ZIKV infection in NHP models. Ultimately, further studies are needed to determine if differences in the magnitude/duration of viremia and clinical signs are the result of animal genotype, virus isolate phenotype, inoculum dose and/or inoculum route.

Unlike ZIKV whose primary transmission mechanism is via mosquito bite, the primary transmission mechanism of HIV-1 is via sexual intercourse. The efficiency of HIV-1 transmission via vaginal or anal intercourse is dependent on a variety of factors such as seminal viral load, number of sex acts, and/or co-infection (48, 49) – likely the case for ZIKV. Similar to other STIs such as HIV-1 (48), our model had a higher number of transmission events from intrarectal inoculation vs intravaginal inoculation. While the per act risk of acquiring HIV-1 infection via vaginal or anal intercourse is low (0.08-1.7%) (48), risk increases proportionally with the cumulative number of sexual acts (49). This trend may be similar for ZIKV, where the cumulative number of sexual acts (low or moderate dose exposure) could increase risk over time. Of note, our experiments were carried out in a controlled research setting where the vagina and rectum of adult macaques was non-traumatically exposed to ZIKV. It is possible that microtears induced during sexual intercourse could further enhance susceptibility to ZIKV infection in human and/or sylvatic NHP populations. Furthermore, sexually transmitted infections (STIs) are known risk factors for increased susceptibility to secondary viral infections via vaginal or anal intercourse (50). Consequently, STIs may increase the likelihood of acquiring ZIKV through the act of vaginal or anal intercourse.

In summary, our results indicate that sexual intercourse is a mechanism for virus transmission in the absence of mosquito-to-human transmission (i.e., effective mosquito control), as well as a mechanism by which ZIKV could be introduced to naïve regions and initiate human-to-mosquito transmission. Furthermore, our findings strengthen the need to avoid risk behaviors, including unprotected male-to-female vaginal intercourse among persons living in regions experiencing ZIKV transmission and for travelers returning from these regions. Moreover, our results indicate the necessity for enhanced diagnostic screening of pregnant women residing in ZIKV-free regions that may have been unknowingly infected with ZIKV through male-to-female vaginal or anal intercourse with an infectious male partner.

FUNDING INFORMATION

This work was supported by a grant from The Defense Advanced Research Projects Agency (DARPA).

DISCLAIMER

The views expressed in this article are those of the authors and do not reflect the official policy or position of the U.S. Department of Defense or the U.S. Army.

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Table 1. Viremia and serological response in rhesus and cynomolgus macaques following intravaginal inoculation of Zika virus.

Macaque	Sex	Viremia* (log_{10} PFU/mL) DPI							Serological response† ($PRNT_{80}$) DPI			
		≤ 2	3	4	5	6	7	≥ 9	7	15	21	28
Rhesus 1	F	–	–	2.2	3.4	2.2	–	–	–	>1:640	>1:640	>1:640
Rhesus 2	F	–	–	–	–	–	–	–	–	–	–	–
Rhesus 3	F	–	–	–	–	–	–	–	–	–	–	–
Rhesus 4	F	–	–	2.2	4.0	2.2	–	–	–	>1:640	>1:640	>1:640
Cynomolgus 1	F	–	–	–	–	–	–	–	–	–	–	–
Cynomolgus 2	F	–	–	–	–	–	–	–	–	–	–	–
Cynomolgus 3	F	–	–	1.0	1.9	3.4	3.5	–	–	>1:640	>1:640	>1:640
Cynomolgus 4	F	–	1.0	1.9	3.4	2.8	–	–	–	>1:640	>1:640	>1:640

DPI, Day post-inoculation.
(-) absence of detectable viremia or serological response.
* Limit of detection 1.0 log_{10} PFU/mL.
† Limit of detection 1:20.

Table 2. Viremia and serological response in rhesus and cynomolgus macaques following intrarectal inoculation of Zika virus.

Macaque	Sex	Viremia* (log_{10} PFU/mL) DPI							Serological response† ($PRNT_{80}$) DPI			
		≤ 2	3	4	5	6	7	≥ 9	7	15	21	28
Rhesus 5	M	–	–		3.8	5.2	3.1	–	–	>1:640	>1:640	>1:640
Rhesus 6	F	–	–	–	–	–	–	–	–	>1:320	>1:640	>1:640
Rhesus 7	M	–	2.9	4.1	3.4	–	–	–	–	>1:640	>1:640	>1:640
Rhesus 8	F	–	2.0	2.9	2.8		–	–	–	>1:640	>1:640	>1:640
Cynomolgus 5	F	–	–	3.2	5.2	5.0	–	–	–	>1:640	>1:640	>1:640
Cynomolgus 6	F	–	–	4.0	4.3	–	–	–	–	>1:640	>1:640	>1:640
Cynomolgus 7	M	–	–	–	3.2	2.1		–	–	>1:640	>1:640	>1:640
Cynomolgus 8	M	2.8	5.0	5.0	3.0	–	–	–	–	>1:640	>1:640	>1:640

DPI, Day post-inoculation.
(-) absence of detectable viremia or serological response.
* Limit of detection 1.0 log_{10} PFU/mL.
† Limit of detection 1:20.

Supplementary Figure 1. Rhesus macaque laboratory values.

Supplementary Figure 2. Cynomolgus macaque laboratory values.